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ORIGINAL PAPER

Effects of KNO_3 pretreatment and temperature on seed germination of *Sorbus pohuashanensis*

BIAN Lei • YANG Ling • WANG Jian-an • SHEN Hai-long

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Abstract: We characterized the effects of KNO_3 pretreatment and germination temperature on dormancy breaking and germination of mature mountain ash seeds. Seeds treated with KNO_3 and germinated at 25 °C followed by 5 °C had significantly higher germination percentages and germination potentials (51% and 49%, respectively), compared with controls. These treated seeds also exhibited reduced germination initiation times (minimum of 48 days), and elevated germination rate indices (up to 97). The germination of seeds subjected to long-term cold storage (2 years at 0–5 °C) was also significantly improved by 3 days of 4% KNO_3 pretreatment before germinating under a variable temperature regimen (5 °C followed by 25 °C, and followed by 5 °C). Germination percentages and germination potentials for these cold-stored seeds reached 67% and 54%, respectively, and the germination rate index increased to 126.99. Pretreatment of mountain ash seeds with KNO_3 represents a practical, effective, and pollution-free method for improving germination, and can be implemented easily within a variety of nursery settings.

Keywords: *Sorbus pohuashanesis*; seed dormancy; seed germination; KNO_3 pretreatment

Introduction

Mountain ash (*Sorbus pohuashanensis* [Hance] Hedl.) is native to northern China, North Korea, and the Russian Far East, and represents a commercially important species within the *Rosaceae* family. This tree is well adapted to withstanding cold stress and can germinate within old-growth spruce forests under low-light conditions and on thick layers of litter. *Sorbus* is a valuable hardwood, prized for its timber and horticultural qualities (Yang et al. 2012; Yang and Shen 2011).

Mountain ash seeds are characterized by an extremely long dormancy period, which complicates seedling cultivation (Shen et al. 2006; Yang et al. 2008a). Cultivation parameters have been extensively studied in a number of closely related species. For example, after-ripening, dormancy, germination, and seedling breeding have been studied in *S. aucuparia* (Basharuddin and Smith 1993; Rogge 1997; Yagihashi et al. 1998; Paulsen and Hogstedt 2002). Cultivation and growth conditions have been examined in *S. tominalis* (Madjidian 2000). The influences of bird foraging on seeds and consequences for germination have been studied in *S. commixta* and *S. aucuparia* (Oster et al. 1987; Yagihashi et al. 1998; Paulsen and Hogstedt 2002). Finally, the influence of temperature on seed germination and dormancy has been studied in *S. glabrescens* (Taylor and Gerrie 1987).

Seed dormancy is controlled by both exogenous (e.g., light, temperature, and moisture) and endogenous factors. The endogenous factors are primarily hormones, but also include small molecules such as reactive oxygen species, hydrogen cyanide, nitric oxide (NO), and alcohols. Dormancy is often broken naturally by the after-ripening process, but it can also be ended artificially through stratification (subjecting seeds to moisture and cold) (Bewley and Black 1994). Mountain ash seed dormancy is highly dependent on temperature (Yang et al. 2008a). The stratification durations that break dormancy in mountain ash are 120 days for newly collected seeds and 105 days for seeds subjected to long-term cold storage (Yang et al. 2008a). The amount of abscisic acid in the seed coat declines considerably during cold stratification (2–5ºC), whereas relative amounts of growth-promoting substances (gibberellic acid in particular) increase in both the seed coat and embryo (Yang et al. 2008b). When seeds are treated with 200 $mg \cdot L^{-1}$ of the synthetic cytokinin 6–benzylaminopurine for 2 days and then incubated at 25 ºC followed by 5 ºC, the initial seed germination time is shortened, and germination percentages and potentials are increased (Yang

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BIAN Lei [1] • YANG Ling[1, 2] (✉) • WANG Jian-an[1] • SHEN Hai-long[1,2]
[1] School of Forestry, Northeast Forestry University, Harbin 150040, P. R. China; [2] State Key Laboratory of Tree Genetics and Breeding, (Northeast Forestry University), Harbin 150040, P. R. China.
E-mail: yangl-cf@nefu.edu.cn

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et al. 2009). However, high prices associated with growth substances that can break dormancy make them impractical for widespread use.

Light is another environmental stimulus that can break dormancy, but it can also inhibit germination. Phytochromes A, B, and E directly induce germination (Bortwick et al. 1954; Shinomura et al. 1996; Hennig et al. 2002). When these photoreceptors are activated, and before they translocate into the nucleus, they initiate a signal-transduction cascade that likely involves G-proteins, cGMP, and the Ca^{2+}/calmodulin complex (Bowler et al. 1994; Mustilli and Bowler 1997). Finally, changes in the sensitivity of seeds to stored and *de novo* synthesized gibberellin seems to complete the germination process (Giba et al. 2006). To our knowledge, the effect of light on seed dormancy and germination in mountain ash has not been examined.

External nitrogenous compounds represent effective substitutes for plant growth substance. Seeds of many crops and tree species exhibit elevated levels of seed viability and germination when treated with nitrogenous compounds (Beligni and Lamattina 2000; Bethke et al. 2004; Gniazdowska et al. 2010). The effects of external nitrogenous compounds on seed germination in mountain ash, however, have not been reported. In this study, we investigated the combined effects of external potassium nitrate (KNO_3) and environmental factors (light and temperature) on mature mountain ash seed germination. Through this research we developed a pre-germination KNO_3 treatment strategy for mountain ash that is practical, effective, pollution-free, and easy to implement within a plant-nursery setting.

Materials and methods

Plant materials

Mountain ash fruits were collected at the Wuying National Forest Park in Yichun City, Heilongjiang Province, China in late September, 2005. Seeds were extracted from the fruit, air-dried, cleaned, and then stored in sealed containers at 0–5 ºC. The mean moisture content of the seeds was 8.4%. The mean 1000-seed weight was (2.42 ± 0.01) g. Mean seed viability, measured by the tetrazolium-staining test (ISTA 1996) was 85.3%.

KNO_3 treatments

Mature mountain ash seeds are received treatments that consisted of soaking in 1%, 2%, 4%, or 6% (w/v) KNO_3 for 1, 2, 3 and then washed. The seeds were then soaked in deionized water for 4 days. The total number of treatments was 12, four KNO_3 concentrations at each of 3 time periods. For each treatment time, internal control seeds were soaked in water for 5, 6, or 7 days.

Germination tests

All seed samples were stirred and sterilized for 2 min in 3×10^4 $\mu L\cdot L^{-1}$sodium hypochlorite solution. After removing the hypochlorite solution, the seeds were rinsed with water. 100 seeds were then placed in 9-cm diameter Petri dishes (each treatment with three Petri dishes, i.e. 300 seeds per treatment). Petri dishes were pre-sterilized with 7×10^5 $\mu L\cdot L^{-1}$ ethanol. A layer of cotton wool was placed inside the dish and covered with a piece of filter paper. The cotton wool and filter paper were soaked with distilled water prior to seed addition.

Control (untreated) and pre-treated seeds were germinated under one of three different temperature/light regimens (Table 1): (1) 5º C under 0.6 $\mu mol\cdot m^{-2}\cdot s^{-1}$ (weak indoor natural light) for the duration of the experiment; (2) 10 days at 5 ºC under weak indoor natural light, followed by 10 days at 25 ºC under a 16-h photoperiod of 40 $\mu mol\cdot m^{-2}\cdot s^{-1}$ light (cool-white fluorescent light), followed by 5 ºC under 0.6 $\mu mol\cdot m^{-2}\cdot s^{-1}$ (weak indoor natural light); or (3) 10 days at 25 ºC under a 16-h photoperiod of cool-white fluorescent light, followed by 5 ºC under 0.6 $\mu mol\cdot m^{-2}\cdot s^{-1}$ (weak indoor natural light), (Table1).

Water was added to the Petri dishes once each day and the percentage of seeds that germinated was recorded 90 and 180 days after initiation of the test. Germination percentage, potentials, and rate index were calculated as follows:

$$G_1(\%) = a\times100/N \tag{1}$$

where, G_1 is Germination percentage; a = the number of germinated seeds on day 180, and N = the total number of seeds.

$$G_2(\%) = a\times100/N \tag{2}$$

where, G_2 is Germination potential; a is the number of germinated seeds on day 90, and N is the total number of seeds. The germination potential is the germination percentage mid-way through the incubation (i.e., day 90).

$$A = \frac{aa_1 + bb_1 + cc_1 + \ldots}{a_1 + b_1 + c_1 + \ldots} \tag{3}$$

where, A is the germination rate index of seeds a, b, c, etc. 1 day after initiating the germination test.

Statistical analysis

Germination was calculated as a percentage of all seeds (viable and dead) in the test. To improve normality and homogeneity of variances, data expressed as percentages were arcsine transformed before analysis. Effects of KNO_3 concentration, treatment time, and the different temperature/light regimens on germination percentage, germination potential, and the germination rate index were analyzed with multivariate analysis of variance (MANOVA), followed by Duncan`s Multiple Range test ($p < 0.05$, or $p < 0.01$). All statistical analyses were performed with DPS (Data Processing System 7.05; Tang and Feng 2002).

Table1. Test design of effects of KNO_3 concentration, treatment time, and the different temperature/light regimens on germination of *Sorbus pohuashanesis* seeds

Treatment	KNO_3 Concentrations (%)	Treatment time (day)			Temperature/light regimens*	Numbers of seeds
		Soaked in KNO_3 (day)	Soaked in water (day)	In total (day)		
1	0 (Control)	0	5	5	(1)	300
2	0 (Control)	0	5	5	(2)	300
3	0 (Control)	0	5	5	(3)	300
4	0 (Control)	0	6	6	(1)	300
5	0 (Control)	0	6	6	(2)	300
6	0 (Control)	0	6	6	(3)	300
7	0 (Control)	0	7	7	(1)	300
8	0 (Control)	0	7	7	(2)	300
9	0 (Control)	0	7	7	(3)	300
10	1	1	4	5	(1)	300
11	1	1	4	5	(2)	300
12	1	1	4	5	(3)	300
13	1	2	4	6	(1)	300
14	1	2	4	6	(2)	300
15	1	2	4	6	(3)	300
16	1	3	4	7	(1)	300
17	1	3	4	7	(2)	300
18	1	3	4	7	(3)	300
19	2	1	4	5	(1)	300
20	2	1	4	5	(2)	300
21	2	1	4	5	(3)	300
22	2	2	4	6	(1)	300
23	2	2	4	6	(2)	300
24	2	2	4	6	(3)	300
25	2	3	4	7	(1)	300
26	2	3	4	7	(2)	300
27	2	3	4	7	(3)	300
28	4	1	4	5	(1)	300
29	4	1	4	5	(2)	300
30	4	1	4	5	(3)	300
31	4	2	4	6	(1)	300
32	4	2	4	6	(2)	300
33	4	2	4	6	(3)	300
34	4	3	4	7	(1)	300
35	4	3	4	7	(2)	300
36	4	3	4	7	(3)	300
37	6	1	4	5	(1)	300
38	6	1	4	5	(2)	300
39	6	1	4	5	(3)	300
40	6	2	4	6	(1)	300
41	6	2	4	6	(2)	300
42	6	2	4	6	(3)	300
43	6	3	4	7	(1)	300
44	6	3	4	7	(2)	300
45	6	3	4	7	(3)	300

***Notes**: (1) 5º C under 0.6 μmol·m^{-2}·s^{-1} (weak indoor natural light) for the duration of the experiment; (2) 10 days at 5º C under weak indoor natural light, followed by 10 d at 25 ºC under a 16-h photoperiod of 40 μmol·m^{-2}·s^{-1} light (cool-white fluorescent light), followed by 5 ºC under 0.6 μmol·m^{-2}·s^{-1} (weak indoor natural light); or (3) 10 days at 25 ºC under a 16-h photoperiod of cool-white fluorescent light, followed by 5 ºC under 0.6 μmol·m^{-2}·s^{-1} (weak indoor natural light).

Results

Germination initiation times after pretreatment with KNO_3

The concentration of KNO_3 did not significantly affect germination initiation time (p=0.23), but the duration of KNO_3 treatment did (p=0.03). Germination initiation time was significantly longer after one day than after three days of KNO_3 pretreatment (p = 0.01), whereas significant differences were not seen between one and two days of pretreatment, or between two and three days of pretreatment. Germination temperatures also affected the germination initiation time (p = 0.001). Although no significant differences were measured between the constant 5 ºC regimen and the 25 ºC to 5 ºC regimen, germination initiation times were significantly longer (60 d; p = 0.001; Fig. 1) when seeds were exposed to the 5 ºC to 25 ºC to 5 ºC regimen.

When interactions between any two of the above three factors were examined (concentration of KNO_3, duration of KNO_3 exposure, and germination temperature) no synergistic effects on germination initiation time were found. In contrast, the combined effect of the three factors significantly affected germination initiation time (p = 0.001).

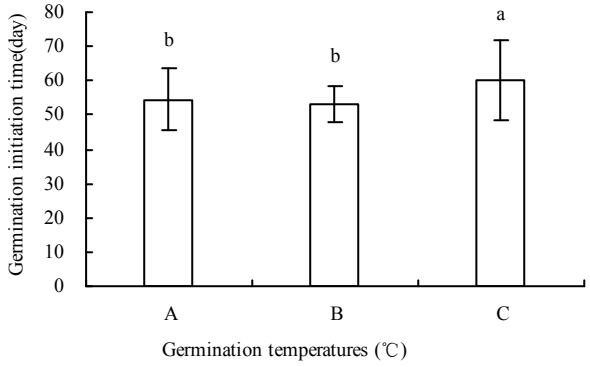


Fig. 1 Effects of germination temperature after KNO_3 pretreatment on germination initiation time. Values are the mean of three independent tests ± the standard deviation. Lowercase letters indicate significant differences ($p \leq 0.01$). Condition A is 5 °C, B is 25 °C followed by 5 °C, and C is 5 °C followed by 25 °C, and followed by 5 °C.

Light intensities did not significantly affect seed germination initiation times. The shortest germination initiation times (48 ± 0 days) were achieved: (1) at constant 5 ºC after 1 or 2 days of 2% KNO_3 treatment, and (2) at variable temperatures (5 ºC followed by 25 ºC, and followed by 5 ºC) after 2 days of 2% or 6% KNO_3 treatment. These germination initiation times, however, were not significantly different from the untreated control (51 ± 6 days; p = 0.37).

Significantly shorter germination initiation times were observed when seeds subjected to long-term cold storage (0–5 ºC for 2 years) were treated with 2% or 4% KNO_3 for two days prior to the variable temperature regimen (5 ºC followed by 25 ºC, and followed by 5 ºC). These stored seeds initiated germination after only 21 days, which was 27 days shorter than unstored seeds subjected to the same treatment.

Germination percentage after pretreatment with KNO_3

MANOVA revealed that all single factors (KNO_3 concentration, treatment time, and germination temperature) affected seed germination percentages ($p \leq 0.01$). In addition, germination percentages were associated with the interaction between treatment time and germination temperature, and the interaction between all three factors ($p < 0.01$). The interaction between KNO_3 concentration and germination temperatures and between KNO_3 concentration and treatment time were not significantly associated with germination percentage.

Duncan's Multiple Range Test showed a highly significant difference in germination percentages between 2% and 4% KNO_3 (p=0.001) and between 1% and 4% KNO_3 (p=0.04), but not between other concentration comparisons (i.e., 1% and 2%, 1% and 6%, 2% and 6%, or 4% and 6% KNO_3) (Fig. 2). The germination percentage of seeds pretreated with KNO_3 for one day was significantly different than those treated for two or three days ($p < 0.01$), regardless of the concentration. No significant differences were seen, however, between seeds treated with KNO_3 for two or three days. When seeds were exposed to the same light intensity (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 10 days, both variable temperature regimens significantly improved the mean seed germination percentage (Fig. 3). In addition, highly significant differences were observed between the different germination temperature regimens ($p < 0.01$; Fig. 3). The highest germination percentage (51.28% ± 4.11%) was achieved with the variable temperature regimen of 25 ºC followed by 5 ºC after two days of 4% KNO_3 exposure. This percentage was significantly different than seen with untreated control seeds (34.08% ± 3.00%; p=0.02). Under the same germination temperature and KNO_3 treatment time, KNO_3 concentration was inversely associated with the mean seed germination percentage (p <0.05; Fig. 2).

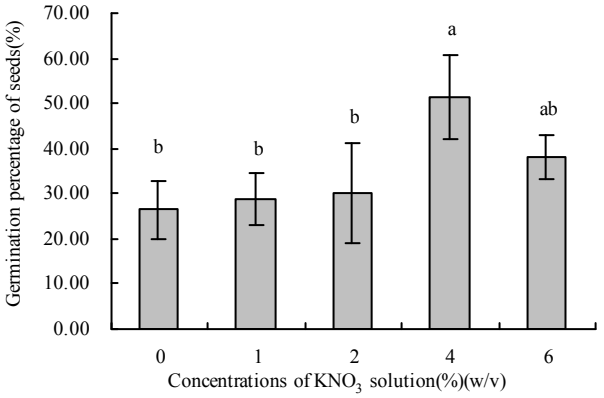
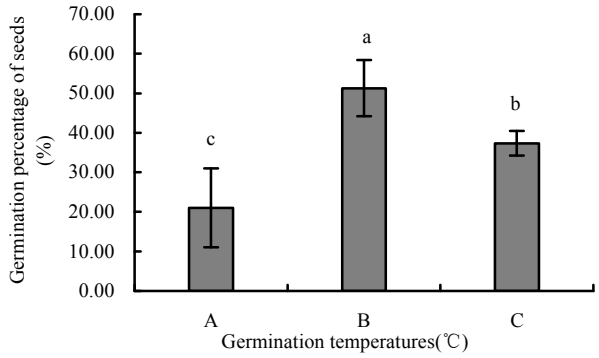


Fig. 2 Effects of KNO_3 concentration on germination percentage. Seeds were treated with different concentrations of KNO_3 for two days before incubation at 25 ºC followed by 5 ºC. Values are the mean of three independent tests ± the standard deviation. Lowercase letters indicate highly significant differences ($p \leq 0.05$).

Significantly higher germination percentages were observed when seeds subjected to long-term cold storage were soaked in 4% KNO_3 for 3 days prior to the variable temperature regimen (5 ºC followed by 25 ºC, and followed by 5 ºC). The percentage of stored seeds that germinated under these conditions was 66.67% ± 5.77%, which represented a 30.02% increase over unstored seeds subjected to the same treatment (51.28% ± 4.11%; p = 0.04).

Germination potential after pretreatment with KNO_3

MANOVA revealed that the interactions between KNO_3 concentration and germination temperatures and between KNO_3 concentration and treatment time had no significant association with the germination potential of seeds, whereas the interaction between the three factors associated with germination potential ($p < 0.05$). In addition, germination potential was affected by the germination temperature, treatment time, and the interaction between treatment time and germination temperature ($p < 0.01$).



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ig. 3 Effects of germination temperature after KNO_3 pretreatment on germination percentage. Values are the mean of three independent tests ± the standard deviation. Lowercase letters indicate significant differences (p ≤0.01). Condition A is 5 °C, B is 25 °C followed by 5 °C, and C is 5 °C followed by 25 °C, and followed by 5 °C.

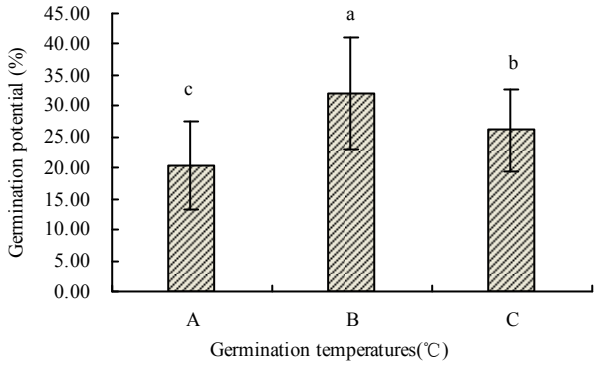
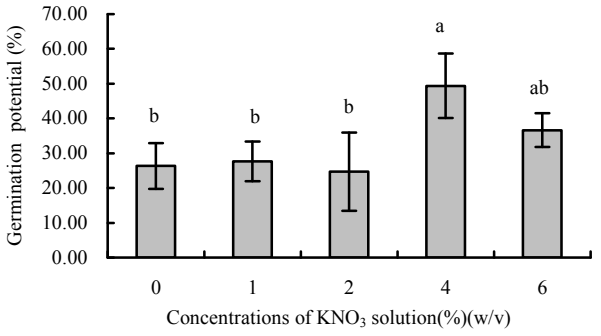


Fig. 4 Effects of germination temperature after KNO_3 pretreatment on germination potential. Values are the mean of three independent tests ± the standard deviation. Lowercase letters indicate highly significant differences (p = 0.01). Condition A is 5 °C, B is 25 °C followed by 5 °C, and C is 5 °C followed by 25 °C, and followed by 5 °C.

The Duncan's Multiple Range Test showed a highly significant difference in germination potential between 1% and 4% KNO_3 and between 2% and 4% KNO_3 ($p < 0.01$), but no significant differences in comparisons between other KNO_3 concentrations. The highest germination potential was achieved with 4% KNO_3. Highly significant differences in germination potential were also found between one day of KNO_3 pretreatment and two or three days of pretreatment ($p < 0.01$). The lowest germination potential was achieved with one day of pretreatment. When seeds were exposed to the same light intensity (40 μmol·m^{-2}·s^{-1}) for 10 days, both variable germination temperature regimens significantly improved mean seed germination potential (Fig. 4).

In addition, there were highly significant differences in germination potential between the three germination temperature regimens (p<0.01, Fig. 4). The highest germination potential (49.37±5.33%) was achieved with the 25 ºC to 5 ºC temperature regimen after 2 days of 4% KNO_3 exposure. This potential was significantly different than seen with untreated control seeds (29.61%±3.44%, p=0.02). Under the same germination temperatures and KNO_3 pretreatment time, KNO_3 concentration was inversely associated with the mean seed germination potential ($p < 0.05$, Fig. 5).



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ig. 5 Effects of KNO_3 concentration on germination potential. Seeds were treated with different concentrations of KNO_3 for 2 days before the 25 ºC to 5 ºC temperature regimen. Values are the mean of three independent tests ± the standard deviation. Lowercase letters indicate highly significant differences (p = 0.05).

The highest germination potential (54.44%±5.77%) was observed when seeds subjected to long-term cold storage were pretreated with 4% KNO_3 for 3 days and then germinated under the 5 ºC to 25 ºC to 5 ºC temperature regimen. This germination potential seemed elevated compared to un-stored seeds subjected to the same conditions (49.37%±5.33%) but the difference was not significant (p = 0.49).

Germination rate index after pretreatment with KNO_3

MANOVA revealed that the KNO_3 concentration, the interaction between KNO_3 concentration and germination temperature, and the interaction between KNO_3 concentrations and treatment time were not significantly associated with the germination rate index of seeds ($p > 0.05$). In contrast, germination temperature,

treatment time, and the interaction between the three factors (KNO_3 concentration, temperature, and treatment time) were highly significantly associated with germination rate index ($p < 0.01$).

The Duncan's Multiple Range Test indicated that the maximum germination rate index was achieved when seeds were treated with KNO_3 for one day. This 1-day germination rate index was significantly higher than that for 2 or 3 days of pretreatment ($p < 0.01$). No significant difference in germination rate index was found between 2 days and 3 days of pretreatment (Fig. 6). Although light conditions did not affect the germination rate index, germination temperature was significantly associated with germination rate index when seeds were exposed to the same light intensity (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 10 days ($p < 0.05$; Fig. 7). Highly significant differences were found between the 25 ºC to 5 ºC temperature regimen and both the constant 5 °C regimen and the 5 ºC to 25 ºC to 5 ºC regimen ($p < 0.01$). Both variable temperature regimens reduced the germination rate index. The maximum germination rate index (97.28 ± 10.81) was achieved when seeds were pretreated with 1% KNO_3 for 1 day and then subjected to the constant temperature (5 °C) regimen. This germination rate index was significantly different than seen with untreated control seeds subjected to the same conditions (maximum 69.63 ± 7.91, p=0.02).

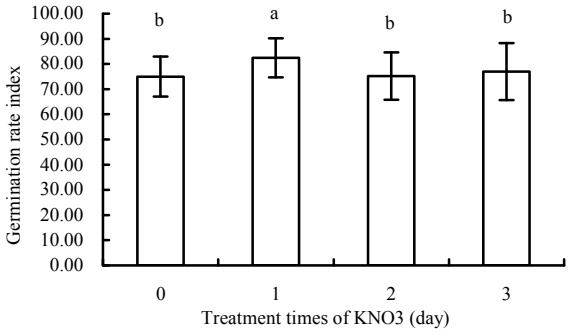

Fig. 6 Effects of KNO_3 treatment time on the germination rate index. Values are the mean of three independent tests ± the standard deviation. Lowercase letters indicate highly significant differences ($p = 0.01$).

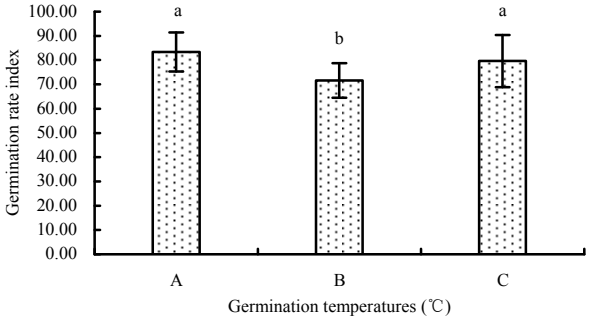

Fig. 7 Effects of germination temperature after KNO_3 pretreatment on the germination rate index. Values are the mean of three independent tests ± the standard deviation. Lowercase letters indicate highly significant differences (p=0.01). Condition A is 5 °C, B is 25 °C followed by 5 °C, and C is 5 °C followed by 25 °C, and followed by 5 °C.

The highest germination rate index (126.9 ±1.56) was observed when seeds subjected to long-term cold storage were pretreated with 4% KNO_3 for 3 days and then germinated under the 5 ºC to 25 ºC to 5 ºC temperature regimen. This germination rate index was significantly higher than measured for unstored seeds subjected to the same conditions (97.28 ± 10.81, p=0.01).

Discussion

Inorganic nitrates significantly increase light sensitivity and decrease the light requirement of seeds (Toole et al. 1955). KNO_3 or gibberellic acid stimulates the germination of *Citrullus lanatus* seeds (Ding et al. 2007), whereas a 1.0% KNO_3 solution increases the germination percentage of *Oriental lily* seeds (Gao et al. 2011). Similarly, *Arabidopsis* seed germination is stimulated by both $NaNO_2$ and $NaNO_3$ (Bethke et al. 2004). In our study, KNO_3 stimulated the germination of mountain ash seeds. The germination levels were affected by both the concentration of KNO_3 and the time of KNO_3 exposure (*i.e.*, treatment time). Gupta et al. (2011) reported a peak germination rate of 96% in *Hippophae salicifolia* when seeds are subjected to a 0.1% KNO_3 pretreatment. They found that higher concentrations of KNO_3 adversely affect seed germination. Suppression of germination by higher concentrations of KNO_3 has also been seen in *Salvia cyanescens* (Yücel and Yilmaz 2009) and *Gladiolus alatus* (Ramzan et al. 2010). We observed a similar trend in this study, as lower concentrations of KNO_3 (1%–4%) increased germination percentages, but higher concentrations (6%) inhibited germination of mountain ash seeds.

Several studies have examined the role NO plays in breaking dormancy and regulating germination (Bethke et al. 2004; Giba et al. 2006; Gniazdowska et al. 2007). NO may be a product of nitrite and nitrate decomposition (Renata and Agnieszka 2006). We speculate that KNO_3 stimulates the germination of mountain ash seeds by the associated production of NO. The proposed connections between KNO_3, NO production, and seed dormancy have not been experimentally confirmed in mountain ash.

Temperature is an important factor during the seed-germination process (Ooi et al. 2012). In this study, incubation at 25ºC followed by 5ºC after KNO_3 pretreatment improved the germination percentage and germination potential of mountain ash seeds. These results are similar to effects of exogenous plant-growth substances and temperature on the germination of mountain ash seeds (Yang et al. 2009). Thus, we propose that a short warm-temperature stratification (< 25 ºC for 10 days) prior to the typical 5 ºC cold stratification could increase germination percentages and germination potential of mountain ash seeds. For seeds subjected to long-term cold storage, however, incubation under the temperature regimen of 5 ºC to 25 ºC to 5 ºC after pretreatment with KNO_3 resulted in significantly higher germination levels than seen with long-term cold-storage seeds incubated at 25 ºC then 5 ºC. We hypothesize that differences in endogenous hormone levels and nutrient concentrations in seeds may alter optimal germination temperatures of mountain ash seeds in these different states (with or without long-term cold

storage). Seed energy reserves are different between newly collected seeds and seeds subjected to long-term cold storage (Qian et al. 2009), and a short, warm stratification may delay ripening of mountain ash seeds. In addition, long-term cold storage may reduce the endogenous abscisic acid levels within seeds, which would stimulate germination (Yang et al. 2008a; 2008b).

Both 6-benzyladenine (Yang et al. 2009) and KNO_3 stimulate the germination of mountain ash seeds. Pretreating seeds with 6-benzyladenine shortens the germination initiation time, increases germination percentage and potential, and reduces the germination rate index of mountain ash seeds. No significant differences between 6-benzyladenine and KNO_3 treatments have been reported ($p > 0.05$). Furthermore, costs associated with KNO_3 are considerably lower than for 6-benzyladenine. Thus, given that cost is a major consideration in the use of pre-germination treatments in large-scale cultivation operations, we propose that the simple, cost-effective pre-germination treatment approach that we describe here be applied to mountain-ash cultivation processes.

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